a transient-change of FRET by ~6% associated with the amperometric spike. Imaging was performed with much lower-time-resolution (~200ms/frame) than the amperometric detection (1ms/point). The fluorescence is thus averaged over each frame, which is equivalent to a 200ms boxcar low-pass-filter, which converts a step change to a linear increase over 200ms reaching 50% of its amplitude at the time of the step. The 50% time point could be determined from the averaged data with a precision <30ms and indicated that the FRET change precedes the upstroke of amperometric spikes on average by ~100ms. These results indicate that the SNAP25 conformational change is associated with fusion pore formation and is not a result of fusion pore expansion. The method is applicable to obtain sub-frame time resolution in imaging if performed simultaneously with high-time-resolution electrochemical or electrophysiological recordings.

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A Combined TIRF/AFM Approach to Investigate Nanomechanical Features at the Cytoplasmic Face of a Plasma Membrane

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The cytoplasmic face of cell membranes has multiple features, such as cortical actin and tethered vesicles, the nanomechanical properties of which are largely unknown. To study these properties, we prepared plasma membrane sheets from a PC12 cell clone (Lang 2008 Meth.Mol.Biology, 440: 51-9) constitutively expressing ANF-EGFP, GFP-tagged proANF (atrial natriuretic factor) (Han et al 1999 *PNAS* 96:14577-82) as a secretory vesicle marker. The membrane sheets are firmly attached to the coverslip with their extracellular face while the cytoplasmic face is exposed. We combined a Nikon Ti-E/B total internal reflection fluorescence (TIRF) microscope with an Agilent PicoPlus atomic force microscope (AFM). Soft AFM cantilevers (Olympus Biolevers) were brought into contact with the exposed membrane face, binding spontaneously to the surface and to secretory vesicles. The membrane sheets and the motion of the cantilever tips were imaged by TIRF microscopy while forces of approximately 50pN or more were applied, pulling the tip away from the surface. At 50 ms time resolution, the noise level of the mechanical measurements was ~1 pN and 0.5 nm (r.m.s.). Dynamic interactions associated with displacements in the 10-40 nm range were recorded. The role of secretory vesicles in this behavior will be investigated.

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Coarse Grain Simulations Reveal Movement of Synaptobrevin C Terminus in Response to Piconewton Forces Suggesting a Novel Fusion Pore Mechanism

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Fusion of neurosecretory vesicles with the plasma membrane is mediated by SNARE proteins, which transfer a force to the membranes. However, the mechanism by which this force transfer induces fusion pore formation is still unknown. The neuronal vesicular SNARE protein synaptobrevin 2 (syb2) is anchored in the vesicle membrane by a single C terminal transmembrane (TM) helix. In coarse grain molecular dynamics simulations self-assembly of the membrane occurred with the syb2 TM helix inserted as expected from experimental data. The free energy profile for the position of the TM domain in the membrane was determined applying harmonic potentials to the peptide in its unbiased position, pulling it towards new biased equilibrium positions. The energy profile determined in this way predicts the energy landscapes for pulling syb2 towards the extravesicular side as expected for SNARE complex zippering. Applying a constant pulling force of 160 pN detaches the synaptobrevin C terminus from the vesicle's inner leaflet lipid head groups within ~100ns and pulls the C terminus deeper into the membrane. This C terminal movement should occur on the physiological millisecond time scale at ~120 pN force. It is facilitated and hindered by specific mutations in parallel with experimentally observed facilitation and inhibition of fusion. These results suggest a mechanism where fusion pore formation is induced by movement of the charged syb2 C terminus into the hydrophobic core of the membrane in response to the force generated by C terminal zippering of the SNARE complex. This displacement of the charged C terminus is expected to destabilize the membrane providing a plausible pathway to fusion pore formation. Supported by NIH grants R01GM085808, R21NS072577, EDICT Project grant 201924, an MRC fellowship and the Wellcome Trust.

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Combination of Amperometry with TIRFM Imaging through a Transparent Electrode Reveals False Fluorescent Transmitter Release Precedes Release of the Granule Core

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We have developed transparent nitrogen-doped diamond-like carbon electrochemical electrodes on glass coverslips for amperometric measurement of exocytosis from an adjacent cell. Since the electrodes are transparent, the bottom of the cell that is adhered to the electrode can be imaged using Total Internal Reflection Fluorescent Microscopy (TIRFM). We labeled vesicles in bovine adrenal chromaffin cells using the fluorescent catecholamine analog FFN 511 such that distinct fluorescent puncta were observed using TIRFM. Upon stimulation with a high K+ solution, fluorescence spots abruptly disappeared presumably due to exocytosis of the FFN dye. Of 45 events analyzed from 12 cells, 43 were accompanied by an amperometric spike measured in the underlying electrode due to oxidation of exocytosed catecholamine. The loss of fluorescence preceded the amperometric spike for 38/43 (88%) of these events, 15 of which had no apparent pre-spike "foot" signal. The interval between the loss of fluorescence and the peak of the amperometric spike was 97 + 9 ms. These results are consistent with the hypothesis that FFN dye is located in the granule halo and is released through a fusion pore before the granule core containing the bulk of the catecholamine is released. In addition, fusion events lacking foot signals presumably still have fusion pores that precede the amperometric spike by tens of milliseconds in order to allow dye escape. Supported by the University of Missouri Research Board.

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Allosteric Model of Ca-Dependent Exocytosis

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Ca-dependent neurotransmitter release is commonly seen as the consequence of an allosteric interaction between one or several Ca-sensors and SNARE-mediated exocytosis. So far, this process has been modeled in terms of a simplified allosteric model, in which a cooperative Ca-binding reaction is coupled to an allosteric transition. Here, I explore classical allosteric models, in which Ca binds independently to several binding sites, however with different affinities and kinetics to the 'relaxed' and 'tense' states, respectively. In particular, I consider cases, in which the binding sites are heterogeneous (e.g. different isoforms of synaptotagmin) and may mix in varying proportions to form a release apparatus. Results are compared to published 'dose response curves' from the Calyx of Held.

1619-Pos Board B389

Cooperative All-Or-None Recruitment of Synaptotagmin C2AB on Single Vesicles Explains Why Ca2+ Regulates the Amplitude of SNARE Mediated Vesicle Fusion

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Synaptotagmin-1 (syt) has been identified as the principle determinant of synchronous release of neurotransmitters at the synapse. The clear correlation between the Ca2+-sensitivity of release and the Ca2+-dependent binding of different syt mutants to negatively charged phospholipid membranes (in the form of small unilamellar vesicles) is in strong favor of this hypothesis.

Both syt wt and C2AB have been exhibiting an enigmatic behavior in all in vitro studies reporting Ca2+regulated vesicle fusion, namely they predominantly, or in some cases exclusively, modulated fusion amplitudes and not fusion kinetics. This means that contrary to intuition syt and Ca2+ do not increase the probability of vesicle fusion. Instead they increase the total fraction of vesicles that are available for fusion through a yet unknown mechanism. Interestingly, this phenotype is reminiscent of the observation that in vivo Ca2+influx in addition to changing fusion kinetics increases the size of the RRP. Here we demonstrate that this striking phenotype originates from the highly cooperative binding of syt to membranes that results to an all-or-none recruitment at the single vesicle level1-4 which is regulated by Ca2+.

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